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Research Article

Lactobacillus acidophilus NCFM preservation through entrapment in soy protein isolate: Persian gum complex coacervates under gastrointestinal conditions in vitro

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ABSTRACT- Probiotics depend on efficient safeguarding during their journey through the gastrointestinal tract, with robust mechanisms guaranteeing their viability and ensuring their beneficial impact once they reach the gut environment. Since the activity and viability of probiotic bacteria are important through gastrointestinal and colonizing conditions, *Lactobacillus acidophilus* NCFM was entrapped in Persian gum (PG) and soy protein isolate (SPI) complex coacervate (CC) to protect and transport them in the gastrointestinal environment. The microorganisms loaded into microcapsules with a SPI: PG ratio of 2:1, were generated with a high encapsulation efficiency of 93.80% using complex coacervation. The study delved into the survival rates of both free and encapsulated cells, as well as their release patterns in simulated gastric and intestinal juices across varied pH levels (1.2, 4, and 7.5), alongside the presence of respective digestive enzymes (pepsin and pancreatin). Results showed that the release rate of bacteria from the capsule was 96.66% after 24 hours. The number of free and encapsulated cells was reduced by 4.03 Log CFU/mL and 2.43 Log CFU/mL in SGJ (pH 1.2), respectively. The findings underscore that the PG-SPI complex coacervate exhibits substantial protection effects against *Lactobacillus acidophilus*.

INTRODUCTION

Probiotics are non-pathogenic microorganisms mostly of human origin and the administration of an adequate amount of them confers a health benefit to the host and can prevent some diseases (Eratte et al., 2017; Lata et al., 2007).

Consumption of probiotic bacteria has some beneficial effects, including increasing intestinal tract health, synthesizing nutrients and improving their bioavailability, decreasing symptoms of lactose intolerance, improving the immune system, decreasing the risk of some cancers, and reducing the prevalence of allergy in susceptible individuals. Activity and viability of probiotic bacteria through gastrointestinal and colonizing conditions are crucial (Tuomola et al., 2001). Additionally, probiotics must maintain high levels of viability and abundance as they traverse from the stomach to the intestine. To this end, the appropriate tolerance to low pH levels and various enzymes and salts is required. Once successfully navigating the stomach, probiotic bacteria encounter further stress upon exposure to pancreatic juices and bile acids. Hence, probiotics must exhibit activity and viability even in the presence of bile acids, notwithstanding the acidity of gastric fluids. The development of delivery systems for probiotic bacteria presents a significant technological challenge. Ensuring probiotics remain viable at the required dosage levels is essential for considering them as "functional ingredients" (Moschakis & Biliaderis, 2017). Scientists studied the improvement of approaches that will increase the viability of probiotics and activity in food formulations, like encapsulation technique, immobilization of cells, prebiotics inclusion, and drying methods (e.g., freeze-drying, spray-drying) (Bosnea et al., 2017; Niakousari et al., 2018).

Microencapsulation of probiotics is considered a practical approach for their efficient survival under gastrointestinal conditions and to improve the viability during shelf life to maintain their health-promoting effects (Niakousari et al., 2018). Microencapsulation techniques for encapsulation probiotic microorganisms are Coacervation/phase separation technique, Extrusion, cold set gels, etc.

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Persian gum (also known as Shirazi gum, Zedo gum, Farsi gum, and Angum gum) is an exudate gum from almond trees (*Amygdalus scoparia* Spach) that usually grows in some parts of Iran. Persian gum has water-insoluble and water-soluble fractions. In recent years, some limited studies have been performed on this gum to provide a deeper insight into the physicochemical properties and structure-function relationships (Hadian et al., 2016; Jafari et al., 2012).

The current study was investigated on encapsulating probiotic *Lactobacillus acidophilus* NCFM within a complex of soy protein isolate and Persian gum, focusing on evaluating cell survivability under *in vitro* gastrointestinal conditions. Additionally, we sought to gauge the protective capabilities of this complex during storage.

MATERIALS AND METHODS

Materials

Persian gum (with an average molecular weight of 4.7 × 10^3 kDa) was provided by Dena Emulsion Company in Shiraz, Iran. Soy protein isolate was obtained from GMAX Co. in China. All chemicals used were sourced from Merck Co. in Darmstadt, Germany.

Preparation of bacteria

Lactobacillus acidophilus NCFM (PTCC No: 1643) was cultured for 48 hours at 37 °C in sterile MRS broth. The acquired culture underwent two rounds of sub-culturing at 37 °C for 2 overnight periods in sterile MRS broth, employing 1% (W/V) inoculum for adaptation and activation as described by Santacruz and Castro (2018). Subsequently, the sample was centrifuged at 11,180 g for 10 min at 4 °C. The bacterial suspension was freeze-dried and kept in a glass tube for further experiments.

Stock solutions and complex coacervate preparation

The obtained cell pellet was reconstituted in 1 mM saline solution (pH 6.80), reaching *Lactobacillus acidophilus* concentrate containing 9.1 Log CFU/mL. The protein solution (1.5% w/v, pH 6.60) was then hydrated overnight (4 °C) and then heated at 80 °C for 30 min, followed by cooling to room temperature. Bacterial suspension (8 mL) was added to 100 mL protein solution. Persian gum (1% w/v, pH 6.60) was dissolved in distilled water and stirred for approximately 3 hours (Kheynoor et al., 2018). Protein solution containing bacteria was stirred and then the polysaccharide solution was added slowly. Then, the pH of the resultant solution was adjusted to 4.6 with HCl (0.1N) to form complex coacervates. The complex coacervates were freeze-dried and kept at 4 °C in a tube for the following experiments.

Bacterial enumeration

The enumeration of viable counts of non-encapsulated *L. acidophilus* NCFM was carried out using MRS agar, following the method described by Silva et al. (2018). To enumerate microencapsulated bacteria, 10 g of microcapsules were dissolved in a saline solution. This peptone water-containing microcapsules was then homogenized (Ultaturax, IKA T18, Germany) at 5,000 rpm for 15 min. Under these conditions, samples were broken, and suspensions (1 mL) were diluted to achieve an appropriate dilution. Colonies were counted after 2 overnight of anaerobic incubation at 37˚C. The viable cell number was expressed as Log CFU per mL of the microcapsule, and the efficiency was determined using Eq. 1:

Encapsulation rate (%) = (total count after encapsulation / total count before encapsulation) × 100 Eq. 1.

Bacterial survival in simulated gastric juice (SGJ) and intestinal juice (SIJ)

The SGJ and SIJ were prepared as described in the method introduced by Hosseini et al. (2019) with some modifications. The pH of saline solution (0.9%) was adjusted at two stages (pH of 1.2 for fast stomach and pH of 4 for breakfast condition) using HCl (1N) and autoclaved. Pepsin was suspended in sterile DDW and filtered by a sterile membrane filter (0.22 µm), then suspended in sterile saline to a final concentration of 0.3% w/v. In brief, 1 g of dried microencapsulated *L. acidophilus* NCFM cells was inoculated into 9 mL of sterile juice solution and maintained at 37 °C under shaking conditions (160 rpm) for 0, 10, 30, 60, and 120 min. Following the incubation period, bacteria were separated from the solutions using centrifugation at 11,000 rpm for 5 min, and the survival rate (%) was calculated using Eq. 2:

Survival rate (%) = (Log CFU N1/Log CFU N0) × 100 Eq. 2.

Where N1 represents the number of viable cells in the microcapsules after treatment with the juice solution and N0 represents the number of viable cells in the microcapsules before treatment. This equation calculates the percentage of viable cells that remain after exposure to the juice solution, measuring the survival rate of the microencapsulated *L. acidophilus* NCFM cells.

Storage stability

To assess storage stability (based on bacterial count), both microcapsules and free bacteria were incubated at 4 °C for 28 days (Nunes et al., 2018).

Biostatistics

The data obtained from all experiments were subjected to statistical analysis using one-way analysis of variance (ANOVA) with a significance level set at $p < 0.05$. Duncan's multiple range tests were conducted using SAS® software (version 9.1, SAS Institute Inc., Cary, NC, USA) to identify significant differences, following the method outlined by Hashemi Gahruie et al. (2017).

RESULTS AND DISCUSSION

Microencapsulation

One of the key parameters highlighting the impact of the encapsulation process and the selected materials is the efficiency of encapsulation (Kheynoor et al., 2018). The

efficiency of loading the cells by coacervation was 93.80%. The average of the *L. acidophilus* cells was 9.11 Log CFU/mL. Dodoo et al. (2017) also used targeted delivery of probiotic bacteria to improve gastrointestinal stability and simulated intestinal colonization. They reported the freeze-dried samples had an efficiency of over 90%, while a complete loss in viability was reported when no protectant was used.

Survival in simulated gastrointestinal condition

One of the most important goals of microencapsulation is to protect the probiotic cells from low pH in the stomach (Nunes et al., 2018). Fig 1 illustrates the count of surviving free cells and microencapsulated *L. acidophilus* under simulated gastrointestinal conditions. pH 1.2 is used to investigate the survival profile on the fast stomach using the substance containing this probiotic. As can be seen, free cells were decreased to 0.89 Log CFU/g of an initial number after 60 min incubation, and this deduction was modeled from a linear graph. After the incubation concluded (120 min), the number of free cells reached 6 Log CFU/mL. In addition, encapsulated cells after being in the same gastrointestinal condition (pH 1.2) and 60 min incubation decreased to 0.91 Log CFU/g of the initial amount. In other words, the number of decreased encapsulated cells was less than that obtained for free cells. After 120 min, the survival rate of encapsulated cells in this pH was 90.10%. Our results showed that the harsh gastric medium significantly affects the survival of all probiotics; however, the encapsulated Lactobacillus in the coacervate structure was more resistant in acidic conditions, most likely because of their adaptation. Similar results were reported by Bosnea et al. (2017). They reported that when probiotics entrapped in complex coacervates were subsequently embedded in calcium alginate gel microspheres, the remaining viable counts at pH 2.0 for 3 h were even higher by almost 1 Log CFU/g.

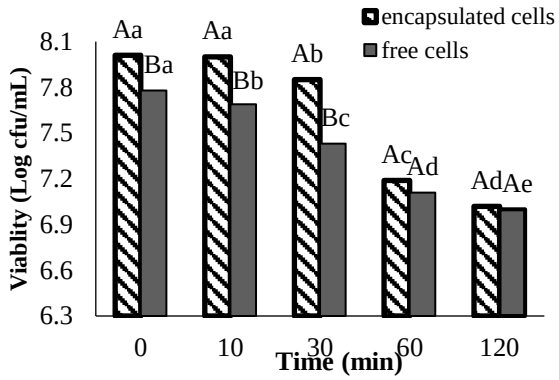

Fig. 1. Survived cell counts of free (Diagonal) and encapsulated cells (Solid) after exposure to simulated gastric juice at 37˚C at pH 1.2. Values shown are means ± standard deviations (n = 3). Different uppercase letters indicate statistically significant differences (P < 0.05) between samples in each time. Different lowercase letters indicate statistically significant differences (P < 0.05) for each sample type between times.

Fig 2 shows the number of survived free and microencapsulated *L. acidophilus* cells in simulated gastrointestinal conditions (pH 4). After incubation for 60 min, the number of free cells decreased to 0.93 Log of an initial number and this deduction was modeled from a linear graph. After 120 min of incubation, the number of free cells reached 7.43 Log CFU/mL. The encapsulated cells decreased to 0.94 Log after subjecting to the gastrointestinal condition at pH 4 and 60-min incubation. In other words, the reduction of encapsulated cell counts was less than that of free cells. The survival rate of encapsulated cells in this pH was 92.95%. It can be concluded that it's better to use the substance containing these probiotic bacteria after a meal, as a dessert or supplement, to increase the survival rate and achieve intestine tract and so more uptake.

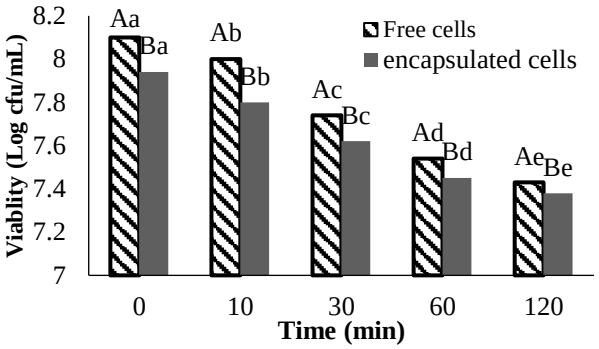

Fig. 2. Survived cell counts of free (Diagonal) and encapsulated cells (Solid) after exposure to simulated gastric juice at 37˚C at pH 4. Values shown are means ± standard deviations (n = 3). Different uppercase letters indicate statistically significant differences (P < 0.05) between samples at each time. Different lowercase letters indicate statistically significant differences (P < 0.05) for each sample type between times.

Release in the simulated intestinal juice

In the next step, the release of encapsulated cells covered in Persian gum/soybean protein isolate was studied (Fig. 3). One of the main goals of this investigation was to find out the state of releasing encapsulated *L. acidophilus* in the condition of the intestine. In the gastric condition with an average pH of 3, both encapsulated and free cells endured for 60 min before transitioning to the small intestine environment with a pH of 7.4, where they were incubated for 24 hours. As depicted in Fig. 3, the count of free cells in the small intestine gradually increased over time, with over 90% release observed after 15 hours, peaking at 96.66% by the 24 hours mark. Banerjee et al. (2017) noted a sudden release of probiotic cells occurring after 50 min when 1 gram of encapsulated bacteria was initially suspended in 10 mL of simulated large intestinal juice. Kim et al. (2008) demonstrated that the viability of non-encapsulated *L. acidophilus* ATCC 43121 decreased from 9.4×10^6 to 1.5×10^6 and 7.1×10^6 to 9.2×10^5 Log CFU/mL, respectively, at bile concentrations of 0.3% w/v and 0.5% w/v during a 24-hour incubation at 37 °C. However, the viability of encapsulated *L. acidophilus* ATCC 43121 remained unaffected at bile concentrations of 0.3% w/v and 0.5% w/v during the same duration.

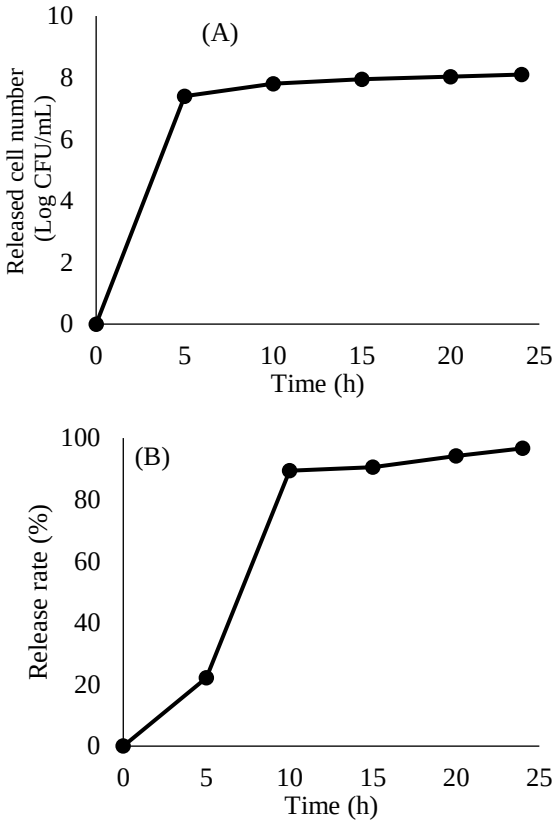

Fig. 3. Release cell count (A) and release rate (B) of encapsulated *Lactobacillus acidophilus NCFM* from FG/SPI in simulated intestinal juice (pH 7.4) after 1 h exposure to SGJ (pH 3.0). Values shown are means ± standard deviations (n = 3).

Viability after exposure to simulated gastric juice during storage

A key parameter of probiotic cultures meant to be used as dietary supplements is that the microorganisms should stay viable during storage (Shu, He, Chen, Song, Cao, & Chen, 2018). For this reason, reductions in the count of viable bacteria throughout the predicted shelf life of a product must be known to some extent and taken as a criterion for selecting such strains. As summarized in table 1, the number of initial encapsulated cells in the first week was less than free cells, but after 4 weeks of different treatments, the number of encapsulated cells was more than that of free cells. Finally, the number of free cells in the condition of SGJ (pH 1.2) changed from 8.03 Log CFU/mL to 4 Log CFU/mL, while the number of encapsulated cells changed from 7.77 Log CFU/mL to 5.34 Log CFU/mL. Moreover, the rate of survival in this pH for free cells and encapsulated ones were 77.12 and 87.90 Log CFU/mL, respectively, which represented the positive effect of encapsulating on *L. acidophilus* surviving in this pH (for empty stomach). Similar results were also observed by testing the survival rate in the condition of breakfast (pH 4), except either free or encapsulated cells had a much higher survival rate in this pH that shows the deduction of the destructive effect of gastrointestinal acid on cells in the condition of full stomach. Therefore, it is recommended that any food or drug containing these probiotics be used after a meal (Zhao et al. 2017).

Table 1. Number of viable encapsulated and free Cells (Log CFU/mL) after 4 weeks.

	Initial cell	Survive cell in SGJ (pH 1.2)	Survival rate (%)	Survived cell in SGJ (pH 4)	Survival rate (%)
Free cells	8.03 ± 0.03[a]	4.00 ± 0.02[b]	77.12 ± 0.01[b]	5.80 ± .0.08[b]	81.86 ± 0.02[b]
Encapsulated cells	7.77 ± 0.04[b]	5.34 ± 0.05[a]	87.90 ± 0.02[a]	6.20 ± 0.06[a]	88.27 ± 0.03[a]

[a,b] Means ± standard deviation (n = 3) with different superscript letters in the same column indicate significant differences ($p \leq 0.05$) among the studied samples.

CONCLUSION

In conclusion, the specified investigation can be considered evidence of the effectiveness of encapsulation in complex coacervate SPI-FG in increasing the shelf life and performance of the *L. acidophilus NCFM* within the gastrointestinal tract environment. The high encapsulation efficiency at around 93% was achieved. The encapsulated cells showed significantly higher survival rates in low pH and simulated GI juice than the free cells. Interestingly, encapsulated cells maintained more than 90% of the survival rate under severe gastric environments compared to free cells. Also, the encapsulated cells had higher survival rates in the presence of bile salt as well as long-term storage, supporting the benefits of encapsulation in enhancing the viability and efficiency of probiotics added to supplements. Based on these results, it is clear that the viability and stability of encapsulated probiotics in essences of dietary supplements and functional food can enhance the positive impacts on the digestive system and gut health, especially in situations where they are used after the consumption of meals.

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CRediT AUTHORSHIP CONTRIBUTION STATEMENT

Conceptualization, SMHH.; methodology, AMK, RN, and MZ.; software, AMK, RN, and MZ.; validation, AMK, RN, and MZ.; formal analysis, AMK, RN, and MZ.; investigation, AMK, RN, and MZ.; resources, SMHH.; data curation, AMK, RN, and MZ.; writing—original draft preparation, AMK, HH, MMN, FG, JBF.; writing—review and editing, AMK, HH, MMN, FG, JBF.; visualization, SMHH.; supervision, SMHH.; project administration, SMHH.; funding acquisition, SMHH.

DECLARATION OF COMPETING INTEREST

The authors declare no conflicts of interest.

ETHICAL STATEMENT

None

DATA AVAILABILITY

The data used to support the findings of this study are available from the corresponding author upon request.

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